IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)
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Babiychuk et al	)
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Serial No.: 09/118276	) Group art unit: 1638
	)
Filed: July 17 , 1998	) Examiner: COLLINS, C.E.
	)
For: METHODS AND MEANS TO MODULATE PROGRAMMED CELL DEATH IN EUKARYOTIC CELLS	)

Declaration under 37 C.F.R. section 1.132

Supplemental Declaration Dr. Marc De Block:

I, Marc De Block, hereby declare that:

1. I am a citizen of Belgium.

2. I received a PhD degree in 1981 from the University of Ghent (Belgium).

3. Since 1984, I have been employed in Ghent, Belgium by PLANT GENETIC SYSTEMS N.V. or its successor firms AVENTIS CROPSCIENCE N.V. and BAYER CROPSCIENCE N.V. ("BCS"). My work currently involves the supervision of projects studying plant growth and development.

4. I am familiar with the field of the plant molecular biology, particularly the fields of cell cycle research, plant development, apoptosis and stress resistance and I have authored and co-authored several scientific publications in these fields (a list of publications has been submitted as ANNEX 1 to my previous declaration in this matter).

5. I am also a co-inventor of the invention described in US patent application 09/118276. (further referred to as "the Application") and I was informed that in the US Official Action, dated February 27, 2002,("Official Action") the Examiner has rejected the pending claims because these claims contain "subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains to make and/or use the invention" and "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had

possession of the claimed invention".

6. It is my understanding that the rejections in the Official Action are based in part on the assumptions that the specification of the application does not support the enablement of the claimed invention for obtaining high vigor in plants and that the data provided in my previous declaration were not convincing.

7. I noticed that Offical Action includes a definition of plant vigor as "a significant increase in plant growth".

8. I further understand that the correlation between the TTC-reducing capacity determined by the assays as described in the application, and plant growth rate or vigor is doubted in the Official Action.

9. I have been informed that the Official Action denies that the claimed nucleic acids have any specific utility.

10. I have also been informed that the Official action contains an objection based on the fact that the specification does not describe the sequence of the *Arabidopsis thaliana* homologue to SEQ ID No 10 (ZAP-type or PARP-1 type).

11. I respectfully disagree on each of these issues, for the reasons elaborated below.

The definition of plant vigor.

12. Plant vigor indeed is mostly apparent from the vegetative growth of a plant. However, the term is also used to refer to agronomic fitness, or agronomic performance, i.e. to the plant's ability to cope with suboptimal growth conditions such as growth under biotic and abiotic stresses. Indeed, the growth of a plant is always the product of its genetic background, combined with environmental factors. It is my opinion plant vigor is best determined by measuring vegetative growth under such suboptimal conditions.

The specification does describe the *Arabidopsis thaliana* homologue to SEQ ID No 10.

13. While it is true that US patent application does not contain a description of the sequence of the *Arabidopsis thaliana* homologue to SEQ ID No 10, at least on pages 43-44 of the specification it is indicated that the *Arabidopsis thaliana* homologue to SEQ ID No 10 can be isolated by hybridization. On page 17, third paragraph to page 18 second paragraph, the specification sets forth methods for isolation of other PARP genes, *inter alia* by hybridization or PCR amplification, particularly using the sequences which encode the so-called PARP signature in the conserved catalytic domain.

14. The *Arabidopsis thaliana* homologue to SEQ ID No 10 has been isolated using as probe a PCR fragment containing the PARP signature (Doucet-Chabeaud et al., Mol. Genet. Genomics 265: 954-963; ANNEX I). The nucleotide sequence of the isolated

gene is available from Genbank under the Accession Nr AJ31705. A comparison of the isolated nucleotide sequence with the sequence of SEQ ID No 10 (ANNEX II) reveals that the A. thaliana homologue has an overall sequence identity of 68.6% to the nucleotide sequence of SEQ ID No 10, but nucleotide stretches of 100 consecutive nucleotides with a sequence identity of more than 75% to the nucleotide sequence of SEQ ID No 10 are present with this nucleotide sequence.

The methods and means described in the specification lead to plants with high vigor.

15. In accordance with Example 3 of the present application, T-DNA vectors were constructed using standard recombinant DNA procedures, comprising a chimeric gene capable of reducing the expression of endogenous PARP genes similar to the constructs described under section 3.1.

    i) I refer to my previous declaration for the construction of T-DNA vectors containing "anti-PARP2" chimeric genes encoding an RNA molecule comprising a complementary sense and antisense region derived from the *Arabidopsis thaliana* PARP gene of the NAP class (SEQ ID No 5; also referred to as APP or PARP-2) either under control of a nopaline synthase promoter (pTYG30) or under the control of the 35S promoter (pTYG29).

    ii) I also refer to my previous declaration for the construction of T-DNA vectors containing "anti-PARP1" chimeric genes encoding an RNA molecule comprising a complementary sense and antisense region derived from the *Zea mays* PARP gene of the ZAP class (SEQ IDs No 1 or 3; also referred to PARP-1) either under control of a nopaline synthase promoter (pTYG34) or of the 35S promoter (pTYG33).

    iii) ANNEX III is a schematic representation of a T-DNA vector containing an "anti-PARP1" chimeric gene encoding an RNA molecule comprising a complementary sense and antisense region derived from the *Arabidopsis thaliana* PARP gene of the ZAP class (also referred to as PARP-1) under control of the 35S promoter (pTYG48).

16. T-DNA vectors were introduced in *Agrobacterium tumefaciens* C58C1Rif (pGV4000) by electroporation as described in Example 3.7 . Plasmid contents of spectinomycin and streptomycin resistant transformants was verified, and the resulting *Agrobacterium* strains were used for transformation of *Arabidopsis thaliana* as well as *Brassica napus.*

17. The T-DNAs comprising the PCD modulating chimeric genes were introduced into *Arabidopsis thaliana* var C24 or Columbia, via *Agrobacterium mediated* transformation essentially as described in Example 4 of the application.

18. The T-DNAs of vectors comprising the PCD modulating chimeric genes were introduced into *Brassica napus* essentially as described in Example 5 of the application.

19. About 30% of the resulting transgenic Arabidopsis lines and oilseed rape plant lines grew more vigorously than the corresponding untransformed oilseed rape plant lines, under suboptimal conditions, such as drought and/or increased temperature.

20. Attached are pictures and data (ANNEX IV) showing increased vegetative growth of Arabidopsis and oilseed rape transgenic lines comprising either "anti-PARP1" (see 15.iii) or "anti-PARP2" (see 15.i) chimeric genes.

    i) Page 1 of ANNEX IV demonstrates the significantly better growth of transgenic Arabidopsis lines containing the chimeric gene of pTYG48, after being subjected to a period of drought.

    ii) Page 2 of ANNEX IV is a graphic representation of the average increase in fresh weight (in mg) of Arabidopsis lines containing either the chimeric gene of pTYG48 (anti-PARP1) or of pTYG29 (anti-PARP2), grown under suboptimal conditions (drought). A clear increase in fresh weight in the transgenic lines compared to the untransformed controls can be observed.

    iii) Page 3 of ANNEX IV, is a graphic representation of the increase in growth under suboptimal conditions of segregating transgenic Arabidopsis populations containing either anti-PARP1 or anti-PARP2 chimeric genes. The plants were germinated from a segregating seed population obtained by selfing transgenic Arabidopsis plant lines. Progeny plants that received the transgene (either homozygous or hemizygous) clearly exhibited a better growth under suboptimal conditions than azygous plants (as determined by PCR) or control C24 plants (green dotted line). This not only confirms that the results observed could be repeated on a large population, but also that the phenotype is linked to the presence of the transgene, and that the PARP expression reducing chimeric gene acts as a dominant gene, as expected.

    iv) Page 4 of ANNEX IV represents a similar experiment for Brassica transgenic plant lines comprising the anti PARP-2 chimeric gene of pTYG29. Here, the plants have been submitted to a cycle of 7 days at 40°C, 2 days at 45°C, a drought period followed by 2 days at 45°C. The plants were germinated from a segregating seed population obtained by selfing transgenic Brassica plant lines. Untransformed control plants and azygous plants (F1 plants which did not receive a transgene from either of the parents) have a much less developed vegetative stage than the hemi and homozygous plants (containing respectively one or two copies of the transgenes). The fresh weight data for the plants of a similar segregating population grown under suboptimal conditions (heat) are graphically represented on page 5 of ANNEX IV.

21. I have also analysed the reduction of the *in vivo* poly(ADP-ribose) polymerase activity in transgenic Brassica napus lines containing the anti PARP-1 chimeric gene of pTYG48. To this end, I used the specific histochemical assay for PARP activity using

biotinylated NAD+ substrate, as described in Bakondi *et al.*, 2002 (J. Histochem. CytoChem. 50(1) pp 91-98). The assay was performed on whole-mounts of hypocotyl explants of control *Brassica napus* plants and transgenic plants, which had been cultured for 5 days and incubated overnight in 100 mg/l acetylsalycilic acid. Whereas a clear PARP activity can be observed in the nuclei of the control line, a clear reduction of PARP activity can be seen in the anti-PARP1 transgenic Brassica line (ANNEX V).

TTC-reducing capacity correlates with plant vigor.

22. I further have been informed the Examiner doubts the correlation between TTC-reducing capacity and vigor or plant growth rate and states that the prior art does not teach such a correlation.

23. In this regard, I would like to refer to WO97/06267 (ANNEX VI) published on February 20, 1997, i.e. before the filing date of the current US application. WO97/06267 (referred to in the current US application page 27, second paragraph) mostly concerns the increase in quality or quantity of genetic transformation using a PARP inhibitor but is also directed to a method to assess the agronomical fitness of a population of plants, (page 15 last paragraph – page 23 first paragraph) using TTC-reducing capacity of plant cells which have been exposed to stress conditions that induce free radical in the tissues or the cells (page 16 first paragraph).

24. Two types of assays based on TTC reducing assays are described in WO97/06267; one test wherein the plant explant is submitted to osmotic stress by incubation into K-phosphate (similar to the test described in Example 6.1 of the current application); and another test wherein a comparison is made between plant explant incubated in the presence of a PARP inhibitor and control plant explants (similar to the test described in Example 6.2 of the current application). Both tests have been described as suitable for determining the quality and agronomical fitness of the plant material (see e.g. page 21 of WO97/06267).

25. Attached are graphics (ANNEX VII) representing the TTC-reducing capacity determined for two lines of the spring variety N90-740 of *Brasssica napus*. In ANNEX VIIA one of the lines had a seed yield comparable to the original N90-740 line and is indicated as the control line. The second line has been determined by field trials as having lower seed yield (about 90% of the control line) and is indicated in the figure as "less vigorous" (lower seed yield and less vigorous growth). As can be seen, the less vigorous line had a lower TTC-reducing capacity. These results have been confirmed for other Brassica lines (ANNEX VIIB; lines A and B had been determined by field trials to be less vigorous than the control line).

26. It is thus clear that within a variety, the comparison of the total TTC-reducing capacity of the different plant lines reflects their vigor. In other words, the TTC-reducing assay as described in the current application can be used to compare the vigor of a transgenic plant having the chimeric genes of the invention capable of reducing endogenous PARP genes, and the untransformed control plants.

Conclusions

27. The methods described in the specification of the patent application do lead to plants with increased vigor, as evidenced by their better growth under suboptimal conditions. PARP activity is reduced in nuclei of the cells of such transgenic plants.

28. The specification provides enough guidance for making plants with increased vigor, without undue experimentation, and for determining plants with increased vigor, using e.g. the TTC-reducing capacity assays.

29. Based on the data set forth in my two declarations, it is my opinion that all the claimed nucleic acids can at least be used to increase vigor in plants. The described methods have a specific utility since they lead to transgenic plants with increased vigor.

I also declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of this application or any patent issued thereon.

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ORIGINAL PAPER

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Ionising radiation induces the expression of *PARP-1* and *PARP-2* genes in *Arabidopsis*

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Abstract By screening for *Arabidopsis* genes activated by ionising radiation (IR)-induced DNA damage, we have isolated a cDNA hybridising with a 3.2-kb mRNA that accumulates rapidly and strongly in irradiated cell suspensions or whole plants. The cDNA codes for a 110-kDa protein that is highly homologous to the 116-kDa vertebrate poly(ADP-ribose) polymerase (PARP-1). It is recognised by a human anti-PARP-1 antibody, binds efficiently to DNA strand interruptions in vitro, and catalyses DNA damage-dependent (ADP-ribose) polymer synthesis. We have named this protein AtPARP-1. We have also extended our observations to the *Arabidopsis app* (*AtPARP-2*) gene, demonstrating for the first time that IR-induced DNA strand interruptions induce rapid and massive accumulation of *AtPARP-1* and *AtPARP-2* transcripts, whereas dehydration and cadmium preferentially induce the accumulation of *AtPARP-2* transcripts. The IR-induced *PARP* gene expression seen in *Arabidopsis* is in striking contrast to the post-translational activation of the PARP-1 protein that is associated with genotoxic stress in animal cells. *AtPARP-1* transcripts accumulate in all plant organs after exposure to ionising radiation, but this is followed by an increase in AtPARP-1 protein levels only in tissues that contain large amounts of actively dividing cells. This cell-type specific accumulation of AtPARP-1 protein in response to DNA damage is compatible with a role for the AtPARP-1 protein in the maintenance of DNA integrity during replication, similar to the role of "guardian of the genome" attributed to its animal counterpart.

Keywords Arabidopsis · Ionising radiation · Gene activation · Poly(ADP-ribose) polymerases · DNA repair

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Introduction

Ionising radiation induces various types of DNA damage (Hutchinson 1985) that need to be removed by DNA repair before cell division, to ensure the transmission of an intact genetic matrix. Depending on the level of DNA damage and the efficiency of repair, the cellular response to irradiation can lead to cell recovery, mutation induction, or alternatively, to the initiation of programmed cell death to specifically eliminate cells that have been too severely damaged (Szumiel 1998). The key function of PARP-1 [Poly(ADP-Ribose) Polymerase-1] in cell recovery from DNA damage induced by IR due to its role in base excision repair (BER) is well understood in animal cells (de Murcia et al. 1998; Oliver et al. 1999; Soldatenkov and Smulson 2000). In plants, however, an understanding of the pathways that lead to recovery from DNA damage is only now emerging.

PARP-1 is a nuclear protein which, when bound to DNA strand breaks, catalyses the formation of branched polymers of poly(ADP-ribose) using NAD^+ as substrate. Polymers of (ADP-ribose) are transferred to a limited number of protein acceptors involved in modulating chromatin architecture or in DNA metabolism, including PARP itself (Oei et al. 1997; d'Amours et al. 1999). The animal 116-kDa PARP-1 protein is composed of three functional modules: the N-terminal DNA binding domain that acts as a molecular nick sensor and is responsible for nuclear location, the central automodification domain that contains the BRCT (BRCA1

C-terminus domain) module involved in the recruitment of enzymes associated with the BER pathway, and the C-terminal catalytic domain, which is by far the most conserved region of PARP. It contains a block of 50 amino acids, referred to as the PARP signature, which forms the catalytic site of PARP, and is conserved virtually unchanged in animals (de Murcia et al. 1994).

In plants, poly(ADP-ribose) polymerase activity (see O'Farell 1995, for review) has been demonstrated by the incorporation of labelled NAD^+ into poly(ADP-ribose) in nuclei of rapidly dividing tissues such as root tips (Payne and Bal 1976), germinating seedlings (Whitby and Whish 1977) and tobacco cell suspensions (Willmitzer 1979). The existence of a 116-kDa protein with similar catalytic properties to those of the animal PARP-1 was first demonstrated in maize nuclei (Chen et al. 1994) and recently the maize PARP-1 was purified and characterised from suspension cultures (Mahajan and Zuo 1998). The amino acid sequence of the maize PARP-1 shows 40–42% identity and about 50% similarity to the known vertebrate PARP-1 sequences. The modular structure of PARP-1, including the DNA binding domain containing two zinc fingers, a putative nuclear localisation signal and the NAD^+-binding domain, is conserved in the maize enzyme.

Members of a new and structurally different class of 72-kDa PARP proteins were first isolated from *Arabidopsis thaliana* (Lepiniec et al. 1995) and maize (Babiychuk et al. 1998). These PARP proteins are significantly shorter, lack the characteristic Zn-finger domains and the nuclear location signal in the N-terminal region, but show striking sequence conservation with the catalytic domain of the vertebrate PARP-1 protein. As expected from the conservation of the catalytic domain, these proteins synthesise (ADP-ribose) polymers and are localized to the nucleus when expressed in yeast – despite the absence of the canonical nuclear location signal that is highly conserved among the PARP-1 proteins (Babiychuk et al. 1998). More recently, the animal counterpart of the plant 72-kDa PARP proteins has been isolated, and DNA damage-dependent activity has been demonstrated for this new mammalian 62-kDa PARP protein, which has been renamed PARP-2 to differentiate it from the classical PARP-1 protein (Amé et al. 1999). This finding suggests the existence of at least two PARP homologues involved in the response to DNA damage in animal cells – PARP-1 (116 kDa) and PARP-2 (62 kDa). The experimental evidence for the involvement of poly(ADP-ribosylation) reactions in DNA repair in plants is provided by the study of homologous recombination in the presence of 3-MB (3-methoxy benzamide), a potent inhibitor of PARP, which increases recombination rates similarly to treatment with DNA-damaging agents (Puchta et al. 1995). Furthermore, the appearance of an 84-kDa PARP-1 cleavage product early after the onset of menadione-induced programmed cell death (PCD) in tobacco protoplasts (Sun et al. 1999), and the inhibition of the apoptotic pathway in cultured soybean cells expressing antisense *PARP-2* mRNA following exposure to severe oxidative stress (Amor et al. 1998) indicate that some of the biological functions of poly(ADP-ribosyl)ation reactions may be conserved between plants and animals.

The present study was initiated to identify the inducible components of the response to IR-mediated DNA damage in *Arabidopsis*. Using RNA fingerprinting, we have isolated a 3.2-kb cDNA that encodes a protein of 983 amino acids (110 kDa), which (1) shows 52% sequence similarity to the 116-kDa vertebrate PARP-1 protein; (2) is recognised by an anti-human PARP-1 antibody; (3) binds to damaged DNA in vitro; and (4) is capable of poly(ADP-ribose) synthesis in the presence of DNase I-treated DNA. We also show that DNA strand breaks induce rapid and massive accumulation of *AtPARP-1* and *AtPARP-2* transcripts in all plant tissues. However, IR-mediated AtPARP-1 protein accumulation occurs only in tissues that contain rapidly dividing cells, indicating a link between AtPARP-1 activity and maintenance of DNA template integrity for replication in *Arabidopsis*. On the other hand, expression of the *AtPARP-2* gene is induced by different kinds of environmental stresses, suggesting an additional role for the AtPARP-2 proteins that is independent of DNA damage.

Materials and methods

Plant material and treatments

A. thaliana (Columbia ecotype) plants were cultivated in a growth chamber under white light on a 16 h light/8 h dark cycle. *Arabidopsis* cell suspensions were grown with constant shaking at 25°C under continuous white light in Sommerville medium as described earlier (Axelos et al. 1992). Individual plants were gamma-irradiated (^{60}Co gamma-irradiator, 2200Tbq; CIS Bio International, France; dose delivery 35 Gy/min) and the tissues were harvested at the indicated times after treatment and stored at –80°C. To induce drought stress, daily watering was omitted for the indicated time periods. Heavy metal stress was induced by the addition of 50 μM $CdCl_2$ to the nutrient medium for the indicated times. Chemical or physical treatments of cell suspensions were applied at the beginning of the exponential growth phase, corresponding to 8–12% PCV (packed cell volume). To induce oxidative stress, 5 mM H_2O_2 was added directly to the culture medium. At the indicated times after treatment, cells were recovered by filtration and stored at –80°C.

RNA techniques

Extraction of total RNA or purified mRNA from plant tissues and cell suspensions was carried out as described previously (Kloppstech et al. 1991; Montané et al. 1997). For Northern analysis, 5-μg aliquots of mRNA were fractionated on an agarose gel and transferred onto a nylon membrane (Amersham). Blots were hybridised for 16 h at 65°C in 0.5 M $NaPO_4$, 5% SDS, 10 mM EDTA. Labelling of specific probes was performed with the Megaprime DNA labelling system (Amersham) using radiolabelled dATP and dCTP. Simultaneous quantification of several transcripts was achieved by multiplex RT-PCR (Godon et al. 1998) with specific primer pairs for *AtPARP-1* (5′-CTGCAACTTCACCTGGGC-3′ and 5′-ACAACTCATGCCCCAACG-3′), *AtPARP-2* (5′-ACAACATCCCCTTGGAGC-3′ and 5′-GGCTTGAGGAACTGAGTGG-3′) and the constitutively expressed *ACT8* gene (5′-GAGATCCACATCTGCTGG-3′ and 5′-GCTGAGAGATT-

CAGGTGCCC-3') as an internal control. The primers were chosen to give PCR products that are easily separated by gel electrophoresis: AtPARP-1, 550 bp; AtPARP-2, 700 bp; and *ACT8*, 300 bp. The PCRs were in the linear range, as attested by comparisons with preliminary calibration reactions.

RNA fingerprinting

The RNA arbitrarily primed PCR (RAP-PCR, Welsh et al. 1992) technique was carried out using a commercial RAP-PCR kit (Stratagene). First-strand cDNAs were synthesised using the Expand Reverse Transcriptase (Roche Diagnostics) with varying amounts (8 ng, 40 ng, 200 ng) of mRNA templates from irradiated or untreated plants and the arbitrary primers provided with the kit. Fingerprinting of mRNA by PCR amplification was performed in the presence of [^{33}P]dATP using the Expand Long Template PCR system (Roche Diagnostics) under the following conditions: 1 cycle of 1 min at 94°C, 5 min at 42°C and 5 min at 68°C; 40 cycles of 1 min at 94°C, 2 min at 60°C and 2 min at 68°C; and 1 cycle of 10 min at 72°C. The PCR products were separated on a 4.5% denaturing polyacrylamide gel and autoradiographed. Candidates for differentially expressed cDNA fragments were eluted from the gel, amplified by PCR, and subcloned using the pCR-Script cloning kit (Stratagene).

Isolation of *AtPARP-1* cDNA

A total of 1.5×10^6 clones from an *A. thaliana* cDNA library (Minet et al. 1992) were screened with the labelled B4 RAP-PCR cDNA fragment under stringent hybridization conditions. One single positive clone, containing an incomplete 2.5-kb *AtPARP-1* cDNA, was isolated and sequenced. The cloning of the missing 5' terminal part was carried out by anchored 5'RACE-PCR (Troutt et al. 1992) using the Marathon cDNA Amplification Kit (Clontech). The template for specific amplification of the *AtPARP-1* cDNA was synthesized from 5 µg of mRNA isolated from irradiated rosettes according to the manufacturer's recommendations. The primers AP1 and AP2, which anneal to the adaptors provided with the kit were used in combination with the nested gene-specific primers RACE1 (5'-GCCCAGAGCACATTGGGCAGAGAGCG-3', pos 906–931), RACE2 (5'-GCACATTGGGCAGAGAGCGAGTGGGC-3', pos 899–929) and RACE3 (5'-TCATGCCATCAGCACATTTATCACGC-3') for the amplification of the 5' *AtPARP-1* cDNA fragment. The longest cDNA fragments obtained by 5'RACE-PCR were subcloned and sequenced to verify that the complete 5' portion of the *AtPARP-1* cDNA had been isolated. Subsequently, the entire *AtPARP-1* cDNA was amplified by using a limited number of PCR cycles on mRNA isolated from irradiated adult rosettes using specific primers defining the 5' and 3' borders of the *AtPARP-1* cDNA, and completely sequenced on both strands. After comparison of the cDNA sequence with the genomic *AtPARP-1* DNA which we also isolated, the cDNA sequence was deposited in the EMBL database under the Accession No. AJ131705.

Characterisation of recombinant AtPARP-1 protein activities expressed in *E. coli*

To express a 6×His-AtPARP-1 fusion protein under the control of the IPTG-inducible Tn5 promoter, the ORF of the *Arabidopsis PARP-1* cDNA lacking the initiator ATG was cloned into pGEM-T (Promega), to yield pGEM-PARP. The *AtPARP-1* cDNA was excised by digestion with *SalI*/*SphI*, and the insert was cloned in frame into the corresponding sites in the pQE-31 expression vector (Qiagen), to yield pQE31-PARP. IPTG dependent bacterial expression of the recombinant AtPARP-1 protein was monitored by SDS-PAGE. The relative amounts of recombinant AtPARP-1 protein in the induced bacterial extracts were estimated by comparison of the AtPARP-1 band with serial dilutions of pure BSA.

For Western analysis, proteins were transferred onto nitrocellulose sheets, and the recombinant *Arabidopsis* PARP-1 protein was probed with a commercial anti-His antibody or an anti-hPARP-1 antibody, using the ECL detection kit (Amersham). The DNA binding capacity of membrane-bound recombinant AtPARP-1 protein was measured as described (Mazen et al. 1989). Activity blot experiments were carried out as previously reported (Simonin et al. 1991), with the exception that blots were incubated at 15°C.

Preparation of protein extracts from plant tissues

Tissue was ground in liquid nitrogen, transferred to 5 vols. of extraction buffer (50 mM Tris-HCl pH 7.5, 4% SDS, 5% β-mercaptoethanol, 5% saccharose and 120 mg polyvinylpyrrolidone per g of tissue), boiled for 3 min and centrifuged twice (10 min, 13,000×*g*). The protein concentration in the supernatant was determined by the Lowry assay (Sigma). For detection of PARP-1 proteins in plant protein extracts, Western analysis was carried out as described above.

Results

Identification of a *AtPARP-1* cDNA by differential screening

We used RNA fingerprinting by RAP-PCR (Welsh et al. 1992) to identify *A. thaliana* transcripts that accumulate strongly in response to IR-induced DNA damage. The pattern obtained by autoradiography from a typical RAP-PCR experiment is shown in Fig. 1. Varying amounts of purified mRNA prepared from irradiated (50 Gy) and untreated adult rosettes were used as templates for RNA fingerprinting with the arbitrary oligonucleotide B4, which yielded an RNA fingerprint band that was obviously present only in RNA preparations from irradiated plants (see the arrowhead in

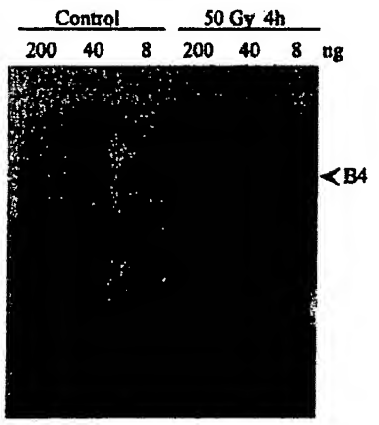


Fig. 1 Identification of an RNA fingerprint that accumulates in response to IR-induced DNA damage. An autoradiograph of a gel displaying RNA fingerprints obtained using the arbitrary primer B4 on 8, 40 or 200 ng of input RNA from unstressed or irradiated rosettes, that were collected 4 h after delivery of a single dose of 50 Gy of gamma radiation. The *arrow* designates a fingerprint that is specifically present in the mRNA population isolated from irradiated rosettes

Fig. 1); this RAP-PCR product was excised from the gel, subcloned and sequenced. A similarity search in the GenBank database, using the longest ORF encoded by the 654-bp B4 cDNA fragment, reveals 61% sequence homology to the C-terminal catalytic domain of human 116-kDa PARP-1, and 56% homology with AtPARP-2 (data not shown). This sequence contains the PARP signature, a stretch of 50 amino acids that is found virtually unchanged in the catalytic site of all PARP proteins, strongly suggesting that this cDNA is derived from a new member of the *Arabidopsis* PARP gene family.

Using the RAP-PCR product B4 to screen an *Arabidopsis* cDNA library prepared from young seedlings (Minet et al. 1992), we isolated a 2.5-kb cDNA that showed complete nucleotide sequence homology with the B4 fragment, but was obviously truncated at the 5′ end, lacking the codons for about 200 amino acids of the N-terminal region that is conserved between the animal PARP-1 proteins. A 1-kb cDNA fragment corresponding to the 5′ end of the *AtPARP-1* cDNA was isolated by 5′RACE-PCR and used to isolate the complete cDNA (see Materials and methods). Genomic DNA sequencing and S1 nuclease protection experiments to determine the transcription start site of the gene further confirmed that we had isolated the complete *AtPARP-1* cDNA (data not shown). The entire cDNA of 3225 bp contains an ORF for 983 amino acids that shows extensive homology to the 116-kDa animal PARP-1 proteins. Alignment of the AtPARP-1 amino acid sequence reveals 60% identity (74% similarity) with the maize PARP-1, and 35–37% identity (51–54% similarity) with most vertebrate PARP-1 sequences (chicken, bovine, human, *Xenopus*, mouse, rat). More importantly, the sequence alignment shown in Fig. 2 clearly demonstrates that the structural motifs and invariant amino acids important for catalytic activity and its function in DNA repair are conserved between hPARP-1 and the plant PARP-1 proteins. Thus, the DNA-binding domain, composed of two zinc finger motifs responsible for specific recognition of DNA strand interruptions (Ménissier-de Murcia et al. 1989; Gradwohl et al. 1990), as well as the critical residues of the bipartite nuclear localisation signal (NLS, Fig. 2) are well conserved. In particular, the three crucial basic amino-acids (K214, R215, K229) in the NLS motif that have previously been shown to be absolutely required for nuclear homing are present in the human and plant PARP-1 proteins (Schreiber et al. 1992).

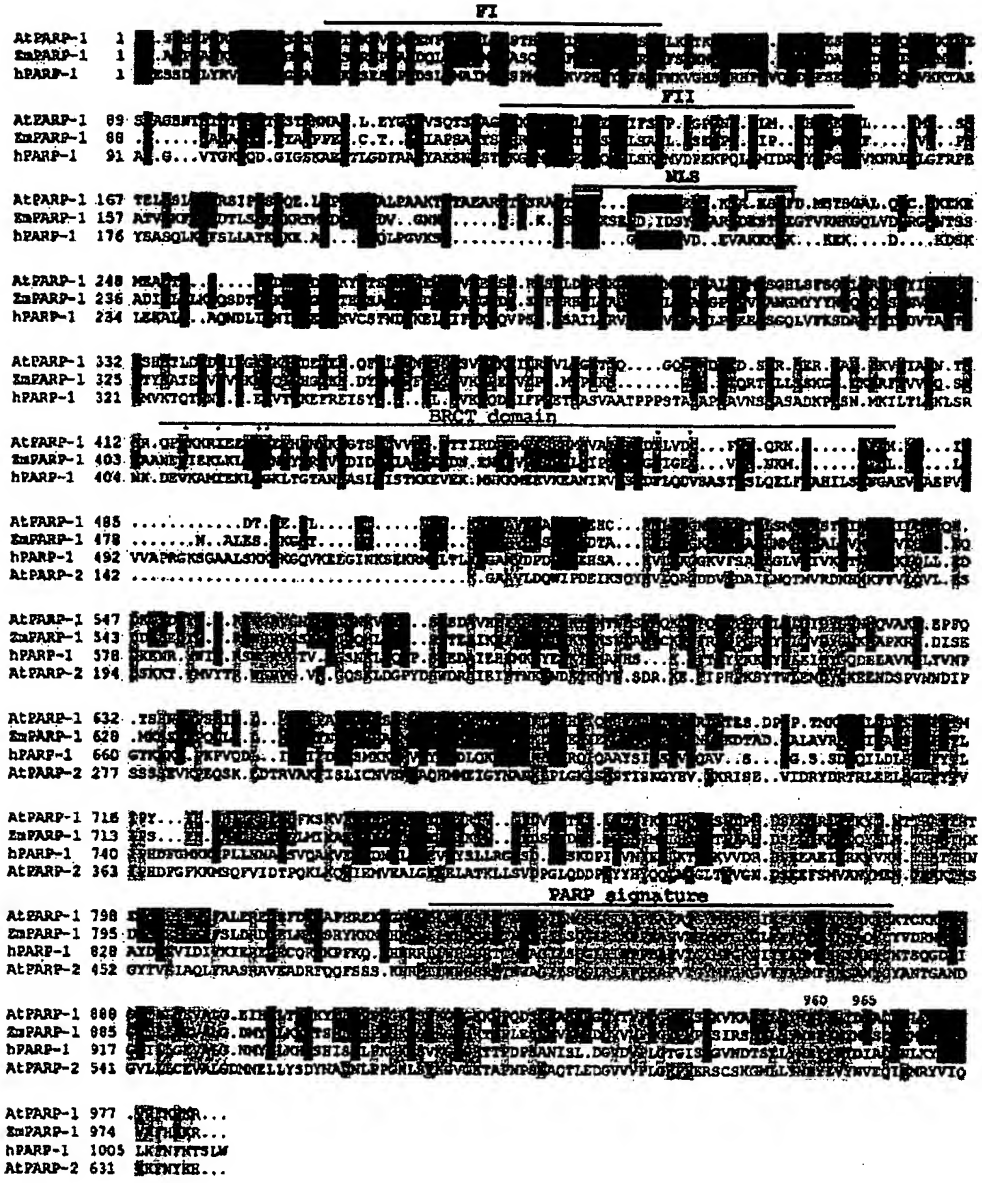
Interestingly, the motif DSVD/N, which is homologous to the defined consensus caspase-3 cleavage site DxxD/N or G (Nicholson and Thornberry 1997) is present in the AtPARP-1, but is absent in the published sequences of the maize PARP-1 homologue. Cleavage of human PARP-1 by caspase-3 at the DEVD/G motif in the NLS, is one of the first steps in the execution programme that irreversibly leads to apoptosis of human cells (Kaufmann et al. 1993). The first demonstration of the biological significance of potential caspase-3-mediated AtPARP-1 cleavage stems from observations made during menadione-induced apoptosis in tobacco protoplasts (Sun et al. 1999), which demonstrate the appearance of an 84-kDa PARP-1 cleavage product, compatible in size with cleavage at the predicted caspase-3 site; this is inhibited by the caspase-3-specific inhibitor AC-DEVD-CHO (N-acetyl-Asp-Glu-Val-aspartinal).

The central automodification domain is the least conserved among all PARP proteins, and seems significantly shorter in plant PARP-1 proteins (see the gaps in the alignment in Fig. 2). However, *Arabidopsis* and maize PARP-1 sequences contain several glutamate acid residues that are required for the automodification reaction, indicating that this function is conserved in the plant proteins. Furthermore, a BRCT module, which is present in a variety of proteins closely related to cell cycle control and DNA repair, is conserved between plant and human PARP-1 proteins (Bork et al. 1996; Callebaut et Mornon 1997). The most highly conserved motif is organised around a conserved aromatic residue (Y466 in AtPARP-1, Fig. 2), the fourth position after the conserved aromatic residue is usually occupied by a cysteine or a serine (C470 in AtPARP-1, Fig. 2). The second highly conserved motif of the BRCT domain consists of two consecutive glycines or glycine-alanine (G424/A425 in AtPARP-1, Fig. 2) preceded in positions –4 and –8 (relative to the first glycine) by hydrophobic residues (I420 and W416 in AtPARP-1, Fig. 2). The BRCT domain is, together with the zinc-finger domain, the major protein-protein contact interface for interaction with partners in the human PARP-1 (Masson et al. 1998). This structural conservation suggests that the function of this domain is conserved in human and plant PARP-1.

The catalytic domain is the most highly conserved among all classes of PARP proteins described to date. In particular, it contains the PARP signature (residues 830–879 in AtPARP-1, Fig. 2), a virtually invariant stretch of 50 amino acids in hPARP-1, the plant PARP-1 and the *Arabidopsis* PARP-2 proteins as outlined in yellow in Fig. 2. This domain also includes the essential residues (indicated in bold) of the donor and acceptor sites that have been defined for the human PARP-1 protein (Ruf et al. 1998), and are conserved in AtPARP-1 (positions 960 and 965 of AtPARP-1 in Fig. 2). By analogy with hPARP-1 therefore, the AtPARP-1 protein possesses all the structural motifs required for nuclear localisation, DNA damage-dependent poly(ADP-ribose) synthesis, and interaction with DNA repair proteins.

Characterisation of the AtPARP-1 protein

To characterise the biochemical properties of AtPARP-1, we overexpressed a recombinant $(His)_6$-AtPARP-1 fusion protein in *E. coli* (see Materials and methods). Coomassie blue staining reveals a protein of the expected molecular weight (about 115 kDa), which is present exclusively in extracts from IPTG-treated



bacteria, and is recognised by the anti-His and anti-hPARP-1 antibodies (Fig. 3, lanes 2–7). This observation underscores the strong sequence and structural conservation between hPARP-1 and AtPARP-1 shown in Fig. 2. Additional bands of lower molecular weight that are detected by the antibodies may correspond to premature translation stops or result from partial protein degradation (Fig. 3, lane 4), as well as from non-specific antibody interactions (Fig. 3, lanes 6 and 7).

To demonstrate the ability of the recombinant AtPARP-1 protein to bind to DNase I-treated DNA, bacterial extracts containing AtPARP-1, or the purified hPARP-1 protein, were blotted onto nylon membranes, renatured and incubated with ^{32}P-labelled, nick-translated DNA. The autoradiograph shown in Fig. 3 (lanes 8–10) demonstrates the presence of DNA strand-break binding activities associated with protein bands of the expected molecular weight in the extracts from bacteria expressing AtPARP-1 and with the purified hPARP-1. This indicates that the poly-histidine extension at the N-terminus of AtPARP-1 does not interfere with the DNA binding activity and also shows that the AtPARP-1 protein has a comparable affinity for damaged DNA in vitro to the purified

Fig. 2 Global sequence conservation and conservation of functional domains between animal and plant PARP proteins. The amino acid sequences of hPARP-1 (H.s., Accession No. P09874), maize PARP-1 (Z.m., Accession No. AF093627), AtPARP-1 (A.th., Accession No. AJ131705) and the catalytic domain of AtPARP-2 (Accession No. Z48243; amino acids 142–637) were aligned using the PileUp program (Genetics Computer Group, Madison, Wis.). The *yellow boxes* indicate amino acids that are strictly conserved in all four sequences, *orange boxes* indicate those conserved in the PARP-1 sequences, *green boxes* those that are conserved in the plant PARP-1 enzymes. Amino acid positions that are conserved in all three plant PARP enzymes are also indicated in *green* in the AtPARP-2 sequence. The conserved zinc fingers (FI and FII), the nuclear localisation signal (NLS), the central BRCT domain, and the PARP signature in the catalytic domain of human PARP-1 are *overlined*. The strictly conserved cysteine and histidine residues in the zinc fingers are *boxed in blue*, the conserved caspase-3 cleavage site in hPARP-1 and AtPARP-1 is *boxed in red*. The conserved amino acid positions within the BRCT domain are indicated by *asterisks and bold letters*. Certain important amino acids in the catalytic site in the AtPARP-1 sequences are also indicated by the *residue number* to clarify the description in the text (see Results)

human protein, since the amount of hPARP-1 loaded on the gel was adjusted so as to be equivalent to the estimated amounts of AtPARP-1 in the bacterial extract (see Materials and methods). To test whether the AtPARP-1 protein expressed in bacteria supports automodification activity, protein extracts were transferred to nylon membranes, renatured, and incubated with ^{32}P-labelled NADH in the presence of activated DNA. The activity blot (Fig. 3, lanes 11 and 12) reveals strong specific labelling of a 116-kDa band, corresponding to the expected size of AtPARP-1, present only in the IPTG-treated extracts. Furthermore, the synthesis of poly(ADP-ribose) polymer in solution is almost completely prevented by the addition of 3-MB, a potent PARP protein inhibitor (data not shown). Taken together, these results suggest that the fundamental biochemical activities associated with the animal PARP-1 proteins are conserved in the plant homologue.

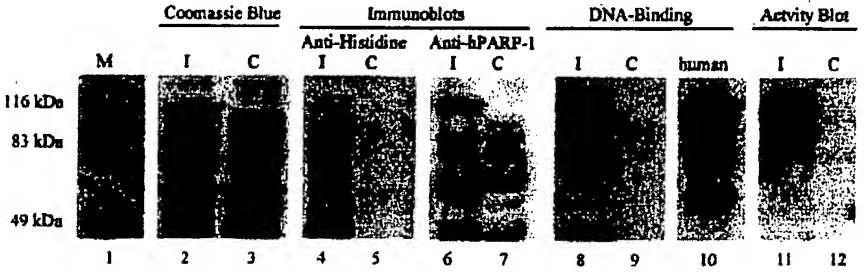
Fig. 3 Characterisation of the AtPARP-1 activity in *E. coli*. The accumulation of full-length recombinant 6×His-AtPARP-1 protein (116 kDa) in extracts from control (C) or IPTG-induced bacteria (I) was followed by Coomassie staining of 12% SDS-PAGE gels (lanes 2 and 3), and Western analysis with anti His-tag (lanes 4 and 5) or anti-hPARP-1 antibodies (lanes 6 and 7). The same extracts and purified human PARP-1 protein were used for Southwestern analysis (lanes 8–10) and activity blot analysis (lanes 11 and 12). Indicated molecular weights are estimated from comigration of coloured molecular weight markers (lane 1)

Ionising radiation induces massive accumulation of *AtPARP-1* and *AtPARP-2* mRNA

The *AtPARP-1* mRNA was identified by screening for plant genes that are activated by IR-mediated DNA damage. To determine whether this observation can be extended to the other *PARP* gene present in the *Arabidopsis* genome, we followed the time course of *PARP* mRNA accumulation in adult *Arabidopsis* leaves following gamma-ray treatment, using the B4 RAP-PCR fragment and the *AtPARP-2* cDNA as probes. The RAP-PCR product B4 hybridises under high-stringency conditions to a single RNA species of about 3.4 kb, as expected from the size of the cloned *AtPARP-1* cDNA. The transcript is rapidly and strongly induced from barely detectable basal levels by exposure to IR. Similarly, the *AtPARP-2* cDNA hybridises to a single 2.2-kb mRNA, which accumulates rapidly and massively after gamma-ray treatment. *AtPARP-1* and *AtPARP-2* mRNA accumulation is transient and dose-dependent, and the mRNA concentrations return to near basal levels 10–12 h after the irradiation (data not shown). Oxidative stress is also capable of damaging DNA, but in contrast to IR the major lesions that result are oxidative base damage and, to a lesser extent, DNA single-strand breaks. We measured by multiplex quantitative RT-PCR the time course of *AtPARP* mRNA induction in *Arabidopsis* cell suspensions after treatment with H_2O_2. As shown in Fig. 4C, the levels of *AtPARP-1* and *AtPARP-2* transcripts seen in untreated cell suspensions remain unchanged for at least 2 h after application of severe oxidative stress, as exemplified by the addition of 5 mM H_2O_2 to the culture medium. Weak induction of *AtPARP* mRNA by oxidative stress is observable only at later time points after treatment, suggesting that DNA strand interruptions rather than oxidative base damage represent the primary signal leading to the observed mRNA accumulation.

Differential regulation of the Arabidopsis *PARP-1* and *PARP-2* genes by environmental stress

We examined the effect of various environmental stimuli, such as UV-B radiation (which preferentially induces



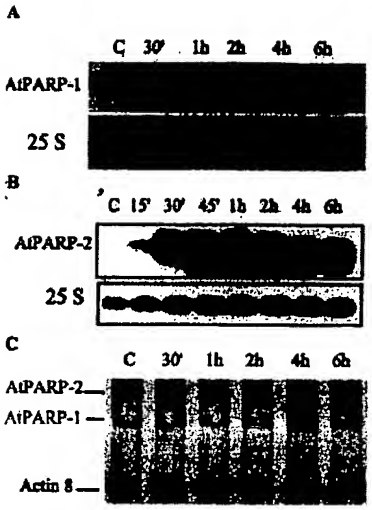


Fig. 4 Time course of *AtPARP-1* and *AtPARP-2* mRNA accumulation after exposure to ionising radiation or oxidative stress. Aliquots (5 µg) of mRNA, prepared from untreated or gamma-irradiated (50 Gy) adult leaves at the indicated times, were used for Northern analysis with *AtPARP-1* cDNA and *AtPARP-2* cDNA as specific probes. Filters were rehybridised with the constitutively expressed *A. thaliana* ribosomal *25S* cDNA as internal control. The time course of *AtPARP-1* and *AtPARP-2* mRNA accumulation in *Arabidopsis* cell suspensions after the application of 5 mM H_2O_2 was followed by quantitative multiplex RT-PCR, including the *Arabidopsis ACT8* mRNA as an internal control

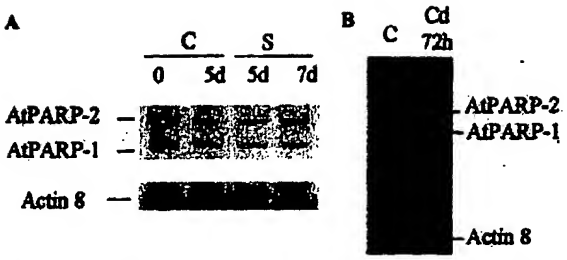


Fig. 5A, B Effect of dehydration and cadmium on the expression of *AtPARP-1* and *AtPARP-2* mRNAs. A Individual plants were cultivated in growth chambers, with (C) or without (S) daily watering, for the indicated times. B Young plantlets were allowed to grow on synthetic nutrient medium without (C) or in the presence of 50 µM $CdCl_2$ for the indicated time. RNA extractions and quantitative RT-PCR with gene-specific primers for *AtPARP-1*, *AtPARP-2* and *ACT8* as an internal control were carried out according to standard procedures

formation of thymidine dimers), dehydration and cadmium on the steady-state levels of *AtPARP-1* and *AtPARP-2* mRNAs. Exposure to increasing cumulative doses of UV-B radiation (up to 30 J/cm^2) did not induce significant changes in *AtPARP* transcript levels in mature leaves (data not shown). Besides H_2O_2, chilling, drought and heavy metal ions also induce oxidative stress in plants (Prasad 1996; Piqueras et al. 1999). We therefore measured the specific levels of *AtPARP-1* and *AtPARP-2* mRNAs in plants that had been submitted to severe water deficit (Fig. 5A) or in young plants that had been cultivated on cadmium-containing medium (Fig. 5B). In both cases we observed no significant changes in the *AtPARP-1* mRNA levels. In contrast, *AtPARP-2* mRNA levels progressively increase with the severity of water deficit. From 5 to 7 days after the onset of dehydration a significant accumulation of the AtPARP-2 transcript is observed. The quantification of induction is hampered by the apparent differences in basal AtPARP-2 transcript levels in individual plants grown under identical conditions. Application of 50 µM Cd^{2+} induces an 8-fold increase in AtPARP-2 transcript levels 72 h after the beginning of the treatment, which corresponds to the time of appearance of necrotic spots on the leaves (data not shown). Our results, together with the increase in the incidence of DNA nicks induced by oxidative stress observed in soybean cell suspensions that express *AtPARP-2* antisense mRNAs (Amor et al. 1998) point to a general role for plant PARP-2 in the response to oxidative stress, while the *AtPARP-1* gene is more specifically activated by DNA strand breaks.

Tissue-specific AtPARP-1 protein accumulation mediated by ionising radiation

To further investigate the tissue-specific distribution of *AtPARP* mRNAs and their IR-dependent accumulation, we performed multiplex RT-PCR reactions with gene-specific primers for *AtPARP-1* and *AtPARP-2*, with *ACT8* as an internal control, on total RNAs prepared from leaves, stems, inflorescences and the root/meristem fraction. *ACT8* mRNAs are constitutively and invariantly expressed in all plant organs analysed, and levels do not change significantly upon irradiation (Fig. 6A). Weak but significant basal levels of *AtPARP-1* and *AtPARP-2* mRNA are detectable in all tissues analysed, where IR induces massive accumulation of both mRNA species; this is consistent with the induction data from mature leaves. To test whether mRNA accumulation is followed by increased protein synthesis, total protein extracts were prepared from the same plant tissues and used for immunoblotting analysis, with the anti-hPARP-1 antibody (Fig. 6B). Although IR induces *AtPARP-1* mRNA accumulation in all plant tissues analysed, the antibody directed against the human PARP-1 protein reveals a protein band of about 115 kDa only in protein extracts from irradiated inflorescences and the root/meristem fraction. Although the interaction of the human antibody with the AtPARP-1 protein may not be sufficiently strong to reveal weak basal AtPARP-1 levels in total protein extracts from untreated plants, the absence of a cross-reacting 115 kDa protein band in irradiated extracts from leaves and stems is unexpected. This may suggest that, depending of the tissue considered, DNA damage-induced *AtPARP-1* transcript levels are not tightly coupled to

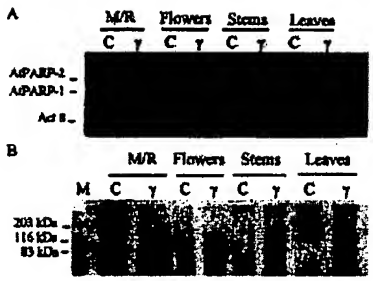


Fig. 6A, B Basal PARP mRNA and protein expression levels and IR-induced accumulation in plant tissues. Individual plants were irradiated (50 Gy), harvested 2 hours after irradiation, and fractionated into the major plant organs. Total RNA or total soluble proteins were prepared from several tissues of untreated (C) or irradiated plants (γ): apical meristem and roots (M/R.), maturing flower buds (flowers), stems, and mature leaves. A Analysis of *AtPARP-1* and *AtPARP-2* mRNA accumulation in plant tissues by quantitative RT-PCR. Reverse transcriptions (RT) and gene-specific PCR amplification were carried out according to standard procedures. B Analysis of AtPARP-1 and AtPARP-2 protein accumulation in plant tissues by Western blotting with anti-hPARP-1 antibody

synthesis of a AtPARP-1 protein that can be detected by the human antibody.

Discussion

In this work, which was initiated in order to characterise *A. thaliana* early-response genes that are differentially expressed after radiation stress, we have isolated a 3.2-kb cDNA that codes for a 110-kDa protein with striking homology to the mammalian 116-kDa PARP-1 protein. The *AtPARP-1* gene is located on chromosome II (TAIR, The Arabidopsis Information Resource), while the previously described *AtPARP-2* gene (Lepiniec et al. 1995) is located on chromosome IV. As expected from the overall conservation of all functional modules, the AtPARP-1 protein has retained the major characteristics of PARP activity, namely binding to nicked DNA, and DNA damage-dependent poly(ADP-ribose) synthesis that is inhibited by 3-MB. Among other important features, the conservation in AtPARP-1 of a BRCT domain is particularly interesting. This protein module which shows limited sequence conservation has been identified by hydrophobic cluster analysis in 24 proteins that are closely related to cell cycle control and DNA repair processes (Callebaut and Mornon 1997): among them are the human PARP-1, XRCC1 and DNA ligase III proteins, all of which are implicated in the BER DNA repair pathway. This domain has since been shown to serve as platform for protein-protein interactions between PARP-1, XRCC1 and DNA ligase III (see Oliver et al. 1999 for review). Thus, the almost superimposable HCA plots (data not shown), together with the conservation in AtPARP-1 of a few amino acids at well-defined positions that are found in all BRCT-containing proteins, suggest that the function of the BRCT module as platform for protein-protein interaction is conserved between plant and human PARP-1 proteins. More particularly, the existence in the Arabidopsis genome of a putative XRCC1 homolog (Accession No. AJ276506.1) showing 53% sequence conservation and 65% similarity with the BRCT domain of human XRCC1 suggests that the association of PARP-1 and XRCC1 is conserved in plants and animals. This is consistent with the involvement of AtPARP-1 in DNA damage repair by the BER pathway. However, we could not find in the *Arabidopsis* genome the structural homolog of DNA ligase III, another major component of the BER pathway. Human DNA ligase III possesses an N-terminal zinc finger domain for specific binding to nicked DNA (Mackey et al. 1999) and a C-terminal BRCT domain for direct interaction with XRCC1 (Nash et al. 1997). This suggests that many, but not all, of the functional components associated with BER may be structurally conserved between plants and human.

The most intriguing result of this work is the rapid and massive accumulation of *AtPARP* mRNA following a large, but sublethal, dose of γ-rays (100% survival). In contrast to mammals, where DNA-damaging agents induce the post-translational activation of the abundant nuclear PARP-1 molecules upon binding to DNA lesions, plants have developed transcriptional regulation as a strategy for regulating PARP activity in response to DNA damage. One of the biological consequences of uncontrolled PARP activity is the intracellular depletion of NAD, which is thought to be a possible signal leading to cell death. If the plant PARP-1 enzyme possesses poly(ADP-ribose) synthesis activity in the absence of DNA damage, the regulation of global PARP activity at the level of gene expression would be a convenient way of preventing excessive consumption of NAD. Previous observations indicated that plant nuclei contain low amounts PARP proteins in contrast to animal cells (Lepiniec et al. 1995; Amor et al. 1998), suggesting that the level of PARP protein necessary to induce DNA repair or apoptosis is regulated at the at the level of transcription in *Arabidopsis*.

In addition to the *AtPARP* genes, the DNA repair gene *RAD51* (Doutriaux et al. 1998), the cell cycle-related gene *AtGR1* (Deveaux et al. 2000) and the *AtLPP1* gene coding for a lipid phosphate phosphatase implicated in the attenuation of phospholipid signalling (Pierrugues et al. 2001), are all rapidly and strongly induced by IR in a dose-dependent manner. This is reminiscent of the bacterial SOS system, and by analogy to the differential activation of about 20 SOS promoters, the transcriptional control of PARP protein biosynthesis may lead to the dose-dependent fine-tuning of a PARP-mediated adaptive response to genotoxic stress: either temporary cell cycle arrest and DNA repair or programmed cell death when cells are too severely damaged. It will be of great interest to elucidate whether the genes mentioned above are members of the same response network under the control of the same transcription factor, as in the case of p53, which plays a central role in the response to IR in animals (Levine 1997).

The coexistence of two independent DNA damage-dependent PARP proteins in animals and plants raises the question of functional redundancy. In this report we show that *AtPARP-1* and *AtPARP-2* mRNAs rapidly and strongly accumulate after IR-induced DNA damage, but only weakly accumulate in response to treatment with 5 mM H_2O_2. Similar doses of H_2O_2 induce PCD in soybean cell suspensions, and this can be significantly reduced by the addition of a PARP inhibitor (Amor et al. 1998). The expression of *AtPARP-2* antisense mRNA leads to an increase in DNA nicks after oxidative stress. Taken together, these data indicate that PARP activity encoded by both plant PARP proteins is necessary for recovery from DNA damage by mediating DNA repair and PCD processes. In addition, our observation that *AtPARP-1* activation by DNA strand interruptions is followed by synthesis of the AtPARP-1 protein only in highly mitotic tissues suggest that sensing of DNA damage and its repair mediated by AtPARP-1 is linked to the maintenance of DNA integrity during replication. This is not a priority in fully differentiated cells that no longer undergo cell division, but need DNA repair for the maintenance of the template for transcription.

On the other hand, plants are subjected in their natural environment to many stress situations that in turn can cause secondary oxidative stress. In many cases the secondary oxidative stress is the major cause of the cellular damage that can occur under unfavourable environmental conditions, including cold stress (Prasad 1996), light stress (Foyer et al. 1994), and pathogen attack (Hammond-Koshak and Jones 1996). The excessive formation of reactive oxygen species in response to the primary environmental stress activates a signal transduction pathway that may be independent of, or function in addition to, the signals induced by the primary stress (Levine et al. 1996). The preferential accumulation of *AtPARP-2* mRNA in response to dehydration or heavy metal stress suggests that the quality of damage is important for specific *PARP* gene induction in *Arabidopsis*, and that *AtPARP-2* rather than *AtPARP-1* may be implicated in additional signal processes that are independent of DNA damage.

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BESTFIT RAW RESULTS
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AtZAP Arabidopsis thaliana mRNA for poly(ADP-ribose) polymerase

to: ZMPARP2:  check: 9035  from: 1  to: 3211

ZAP2 Zea mays mRNA for poly(ADP-ribose) polymerase (3211bp)

Symbol comparison table: /san/data/public/netgenics/datafiles/SmithWaterman
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Default scoring matrix used by BESTFIT for the comparison of nucleic
acid sequences.  BESTFIT uses the method of Smith/Waterman to make alignments.
X's and N's are treated as matches to any IUB ambiguity symbol. All
mismatches for IUB symbols are -9, making BESTFIT clip off the best
fitting segment at the point where the sequences stop being very similar.

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ATH131705= x ZMPARP2: August 6, 2002 09:26  ..

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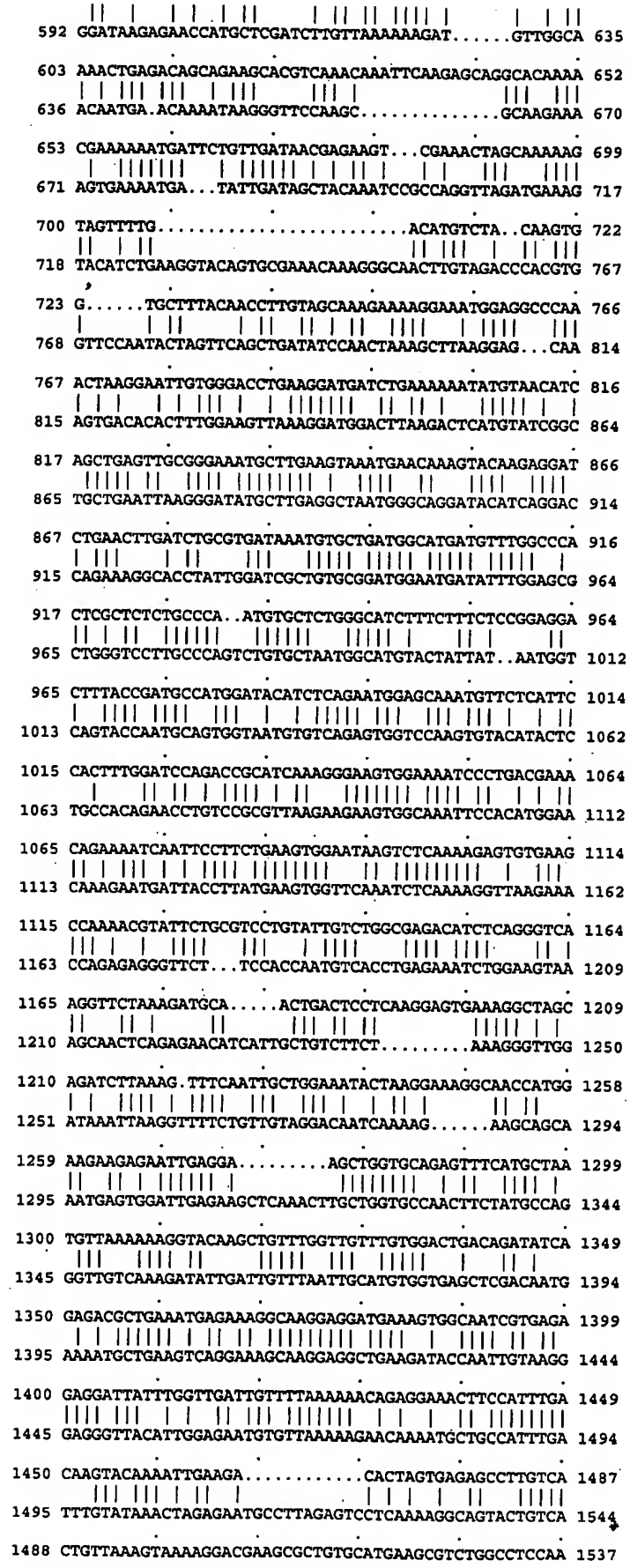
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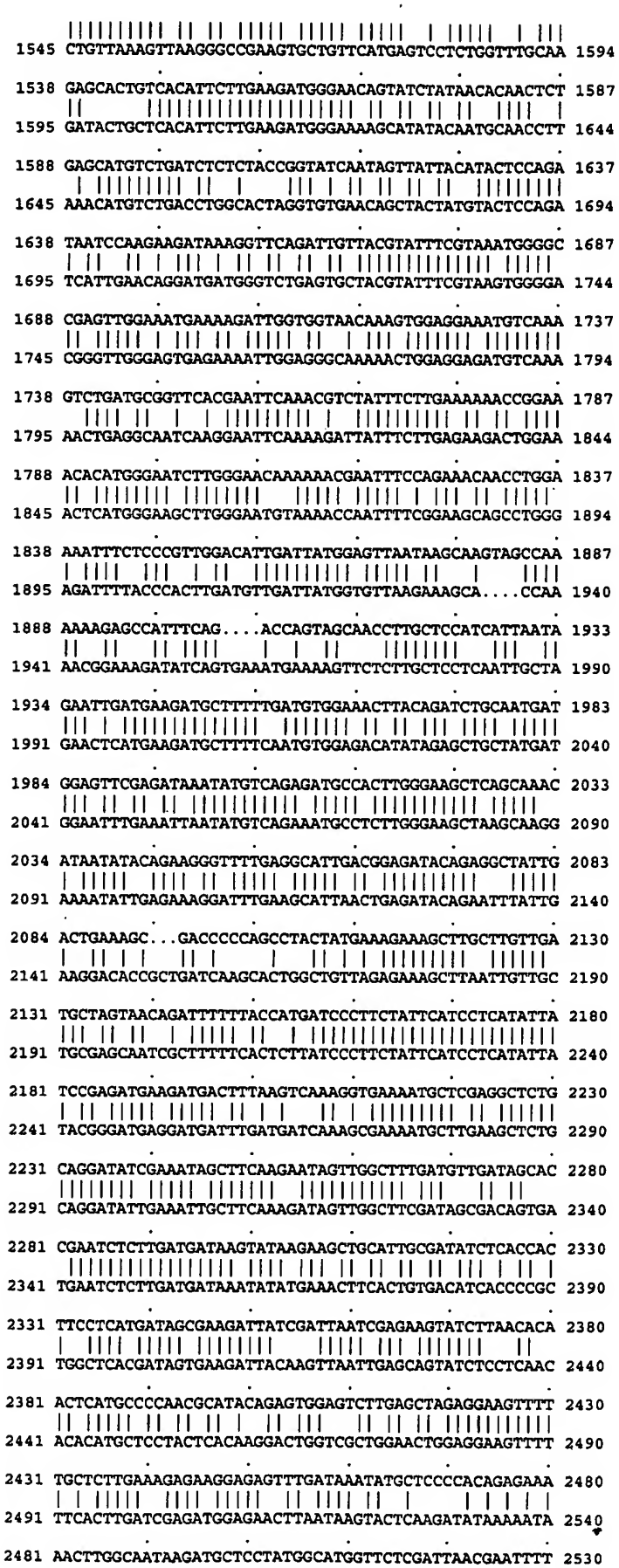

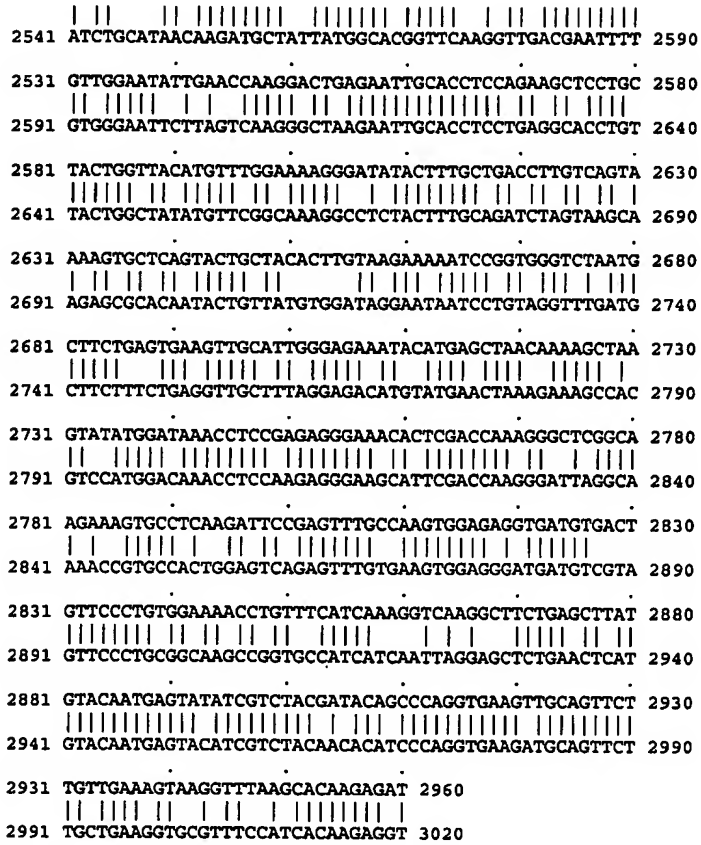
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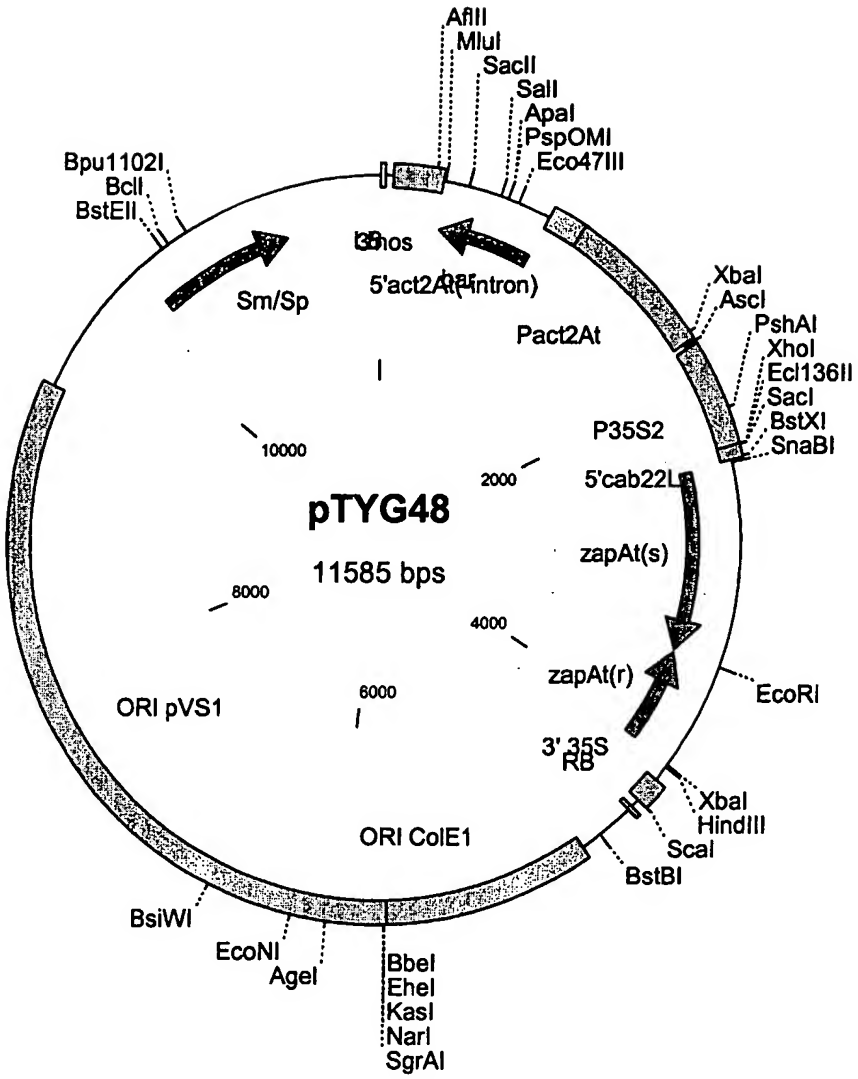


De Block
Supplemental Declaration
ANNEX III

Molecule: pTYG48, 11585 bps DNA Circular
File Name: ptyg48.cm5, dated 06 Aug 2002

Description: LB-3'nos-bar-5'act2At(-intron)-Pact2At-P35S2-5'cab22L-zapAt(s)-zapAt(r)-3'35S-RB

Notes: Author:: Yannick Gansemans
Notebook:: 0359

Molecule Features:

Type	Start	End		Name	Description
REGION	1	25		LB	Left T-DNA border (synthetic)
REGION	324	65	C	3'nos	3' UTR from A. tumefaciens nopaline synthase
GENE	895	344	C	bar	Basta resistance gene from Streptomyces speci
REGION	1073	896	C	5'act2At(-intron	5' UTR without intron from A. thaliana Actin-
REGION	1836	1074	C	Pact2At	Promoter from A. thaliana Actin-2 gene
REGION	1863	2396		P35S2	Promoter from CaMV 35S gene
REGION	2397	2466		5'cab22L	5' UTR from Petunia Chlorophyl a/b binding p
GENE	2483	3524		zapAt(s)	Arabidopsis Zn-finger type PARP, sense fragme
GENE	4069	3541	C	zapAt(r)	Arabidopsis Zn-finger type PARP, reversed fra
REGION	4175	4309		3' 35S	3' UTR from CaMV 35S gene
REGION	4353	4377		RB	Right T-DNA border (synthetic)
REGION	4677	5740		ORI ColE1	Plasmid replication origin for stable mainter
REGION	5741	9511		ORI pVS1	Plasmid replication origin for stable mainter
GENE	10237	11028		Sm/Sp	aadA gene from ???

Drought tolerance of Arabidopsis lines

sow seed

↓ *17 days*

stop watering

↓ *4 days*

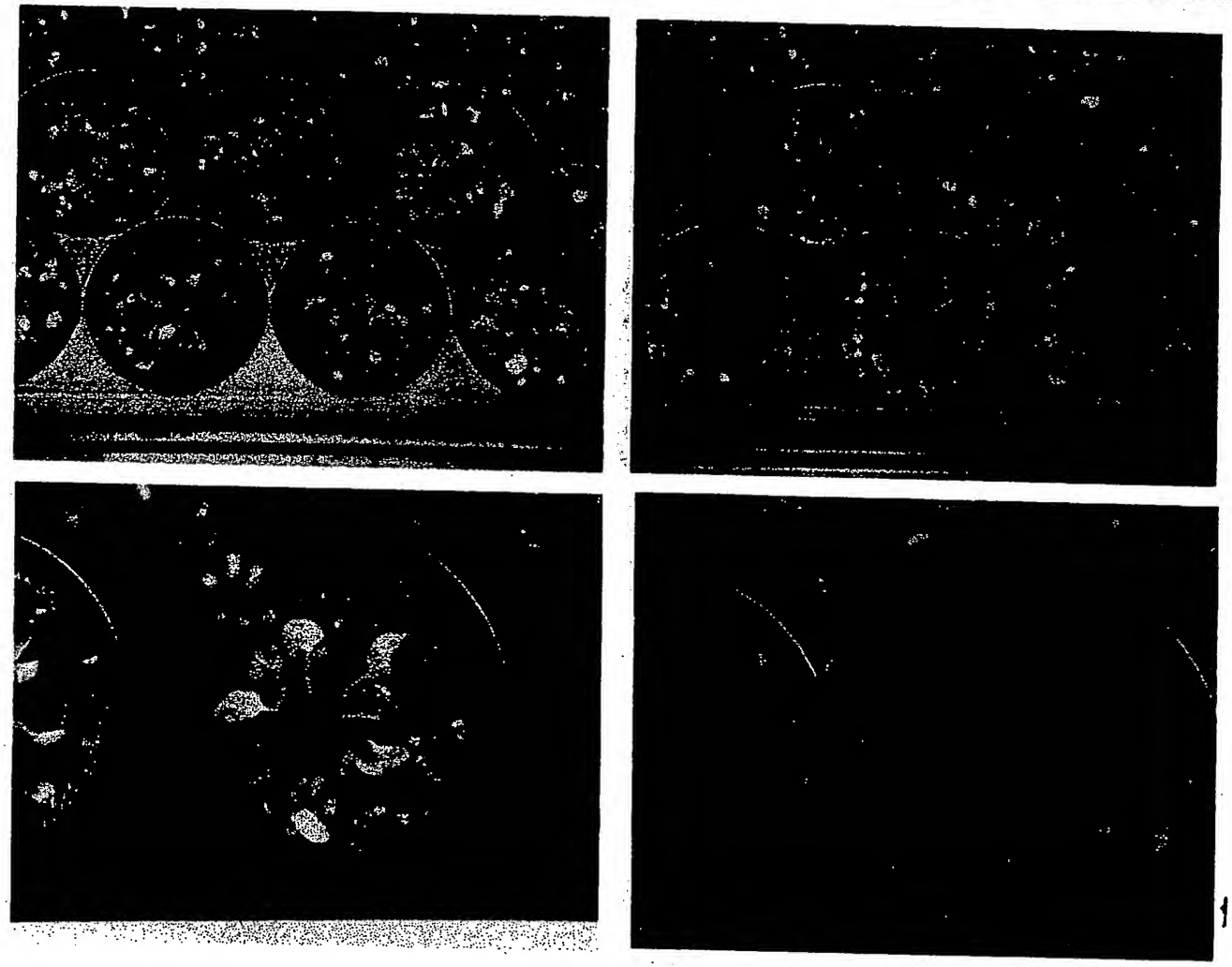
800ml water/tray

↓

no watering

↓ *7 days*

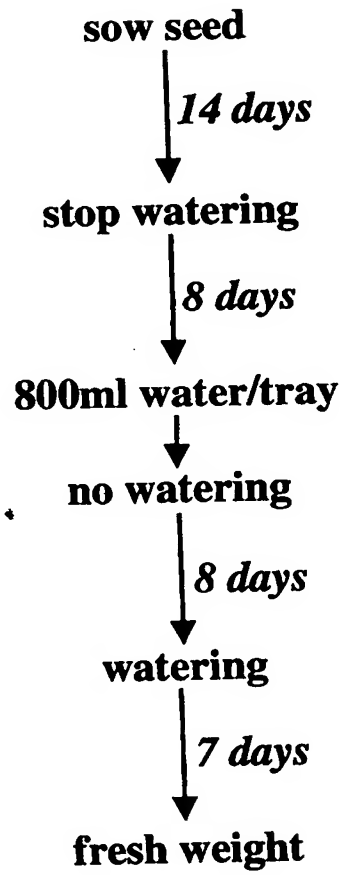
picture

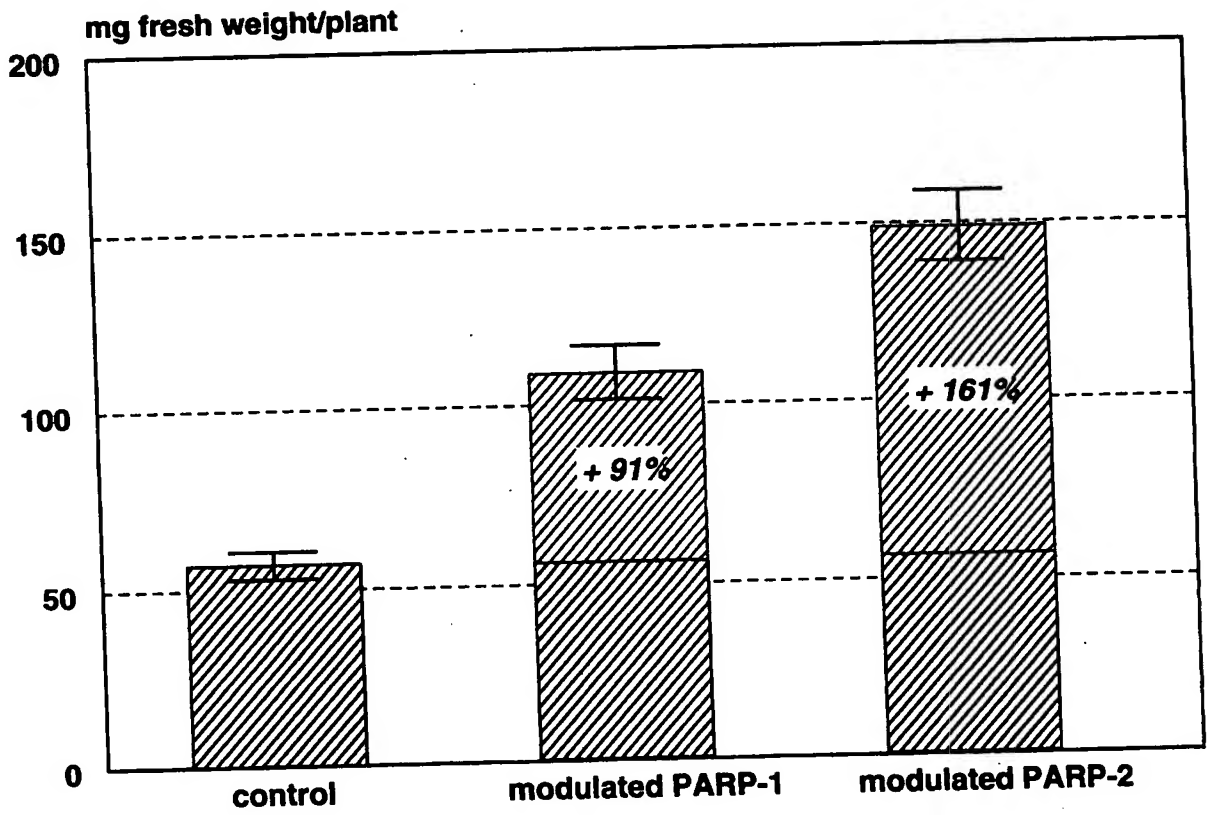


control

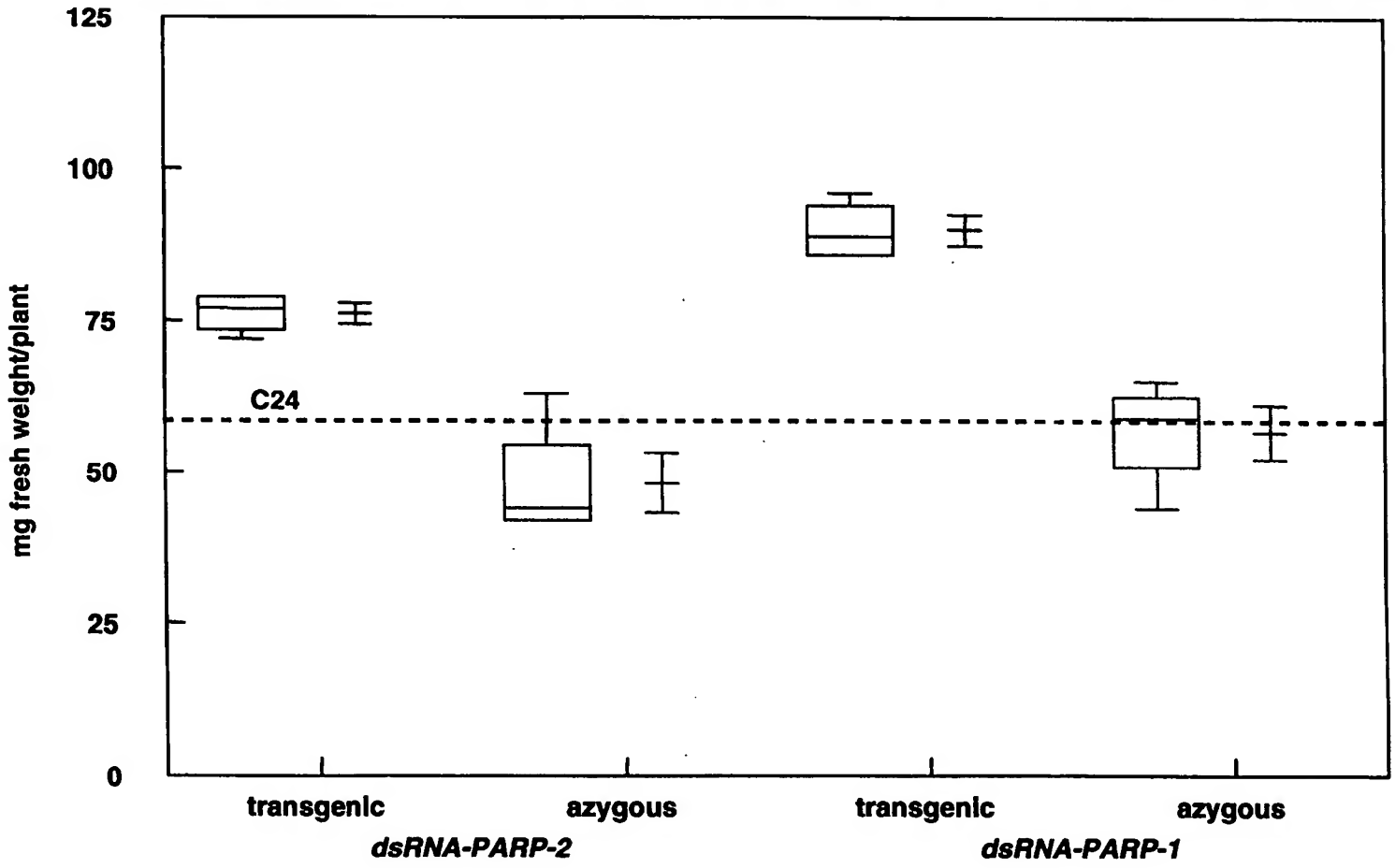
p35S:anti-PARP-1

Influence of modulated PARP-activity on growth of Arabidopsis plants under drought stress





Drought tolerance of Arabidopsis lines segregating for dsRNA-PARP-1 or dsRNA-PARP-2



Co-segregation of stress tolerance and the anti-PARP-2 gene in *Brassica napus*

sow seed

↓ *10 days*

40°C

↓ *7 days*

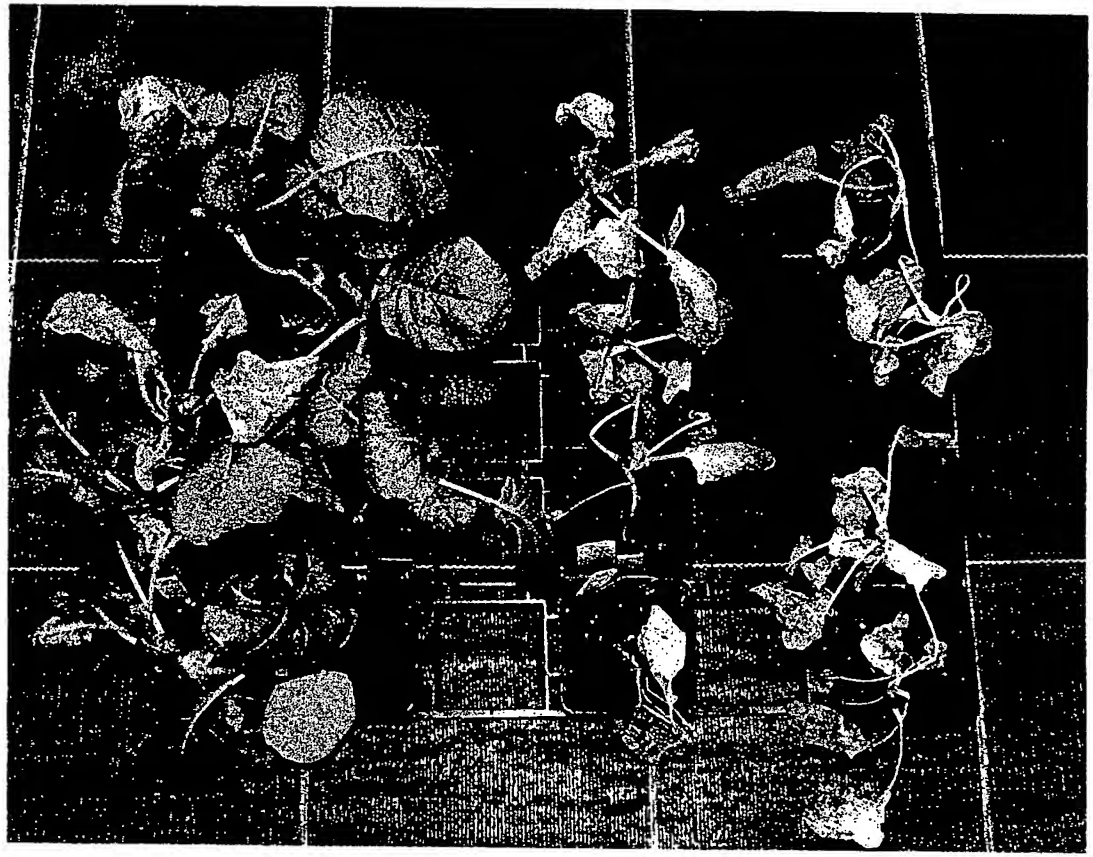
45°C

↓ *2 days*

drought + 45 °C

↓ *2 days*

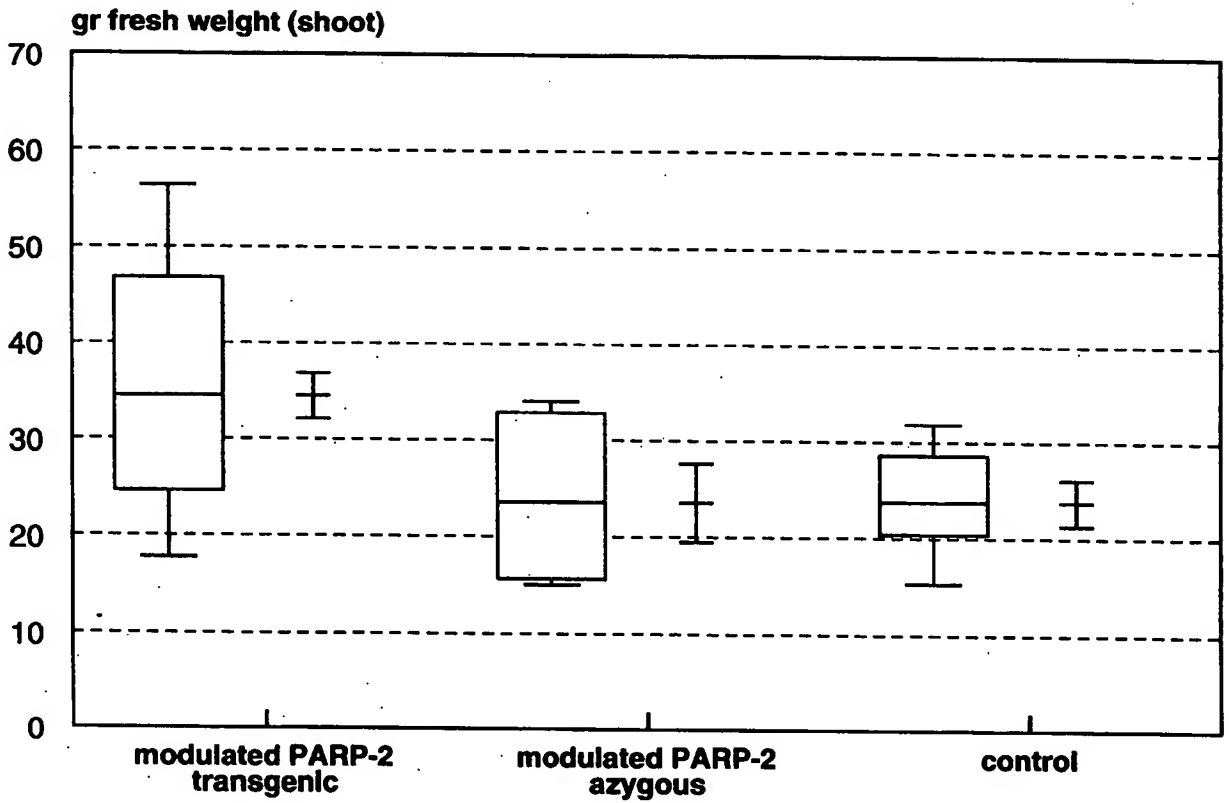
picture



hemi- + homozygous | azygous

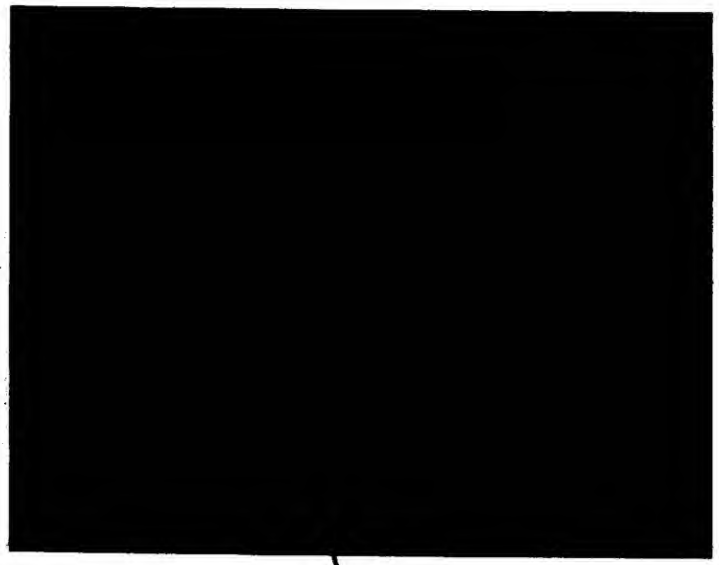
anti-PARP-2 line | control line

Fresh weight *Brassica napus* plants treated for three weeks at 40 °C



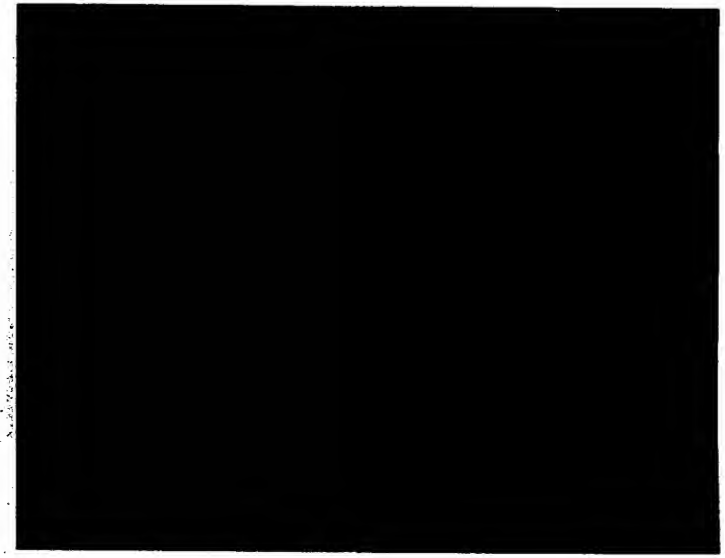
Whole mount PARP-assay on hypocotyl explants of Brassica napus using biotinylated NAD^+

Control



labeled nucleus

dsRNA-PARP-1 line



Explant material: 5 days cultured hypocotyl explants
Stress condition: overnight incubation in 100mg/l aspirine

De Block
Supplemental Declaration
ANNEX V

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, 15/79, 5/10, A01H 5/00 — A2

(11) International Publication Number: WO 97/06267

(43) International Publication Date: 20 February 1997 (20.02.97)

(21) International Application Number: PCT/EP96/03366

(22) International Filing Date: 31 July 1996 (31.07.96)

(30) Priority Data:
95401844.6 4 August 1995 (04.08.95) EP
(34) Countries for which the regional or international application was filed: GB et al.

(71) Applicant *(for all designated States except US)*: PLANT GENETIC SYSTEMS, N.V. [BE/BE]; Jozef Plateaustraat 22, B-9000 Gent (BE).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: DE BLOCK, Marc [BE/BE]; Abrikozenstraat 26, B-9820 Merelbeke (BE).

(74) Agents: GUTMANN, Ernest et al.; Ernest Gutmann - Yves Plasseraud S.A., 3, rue Chauveau-Lagarde, F-75008 Paris (FR).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

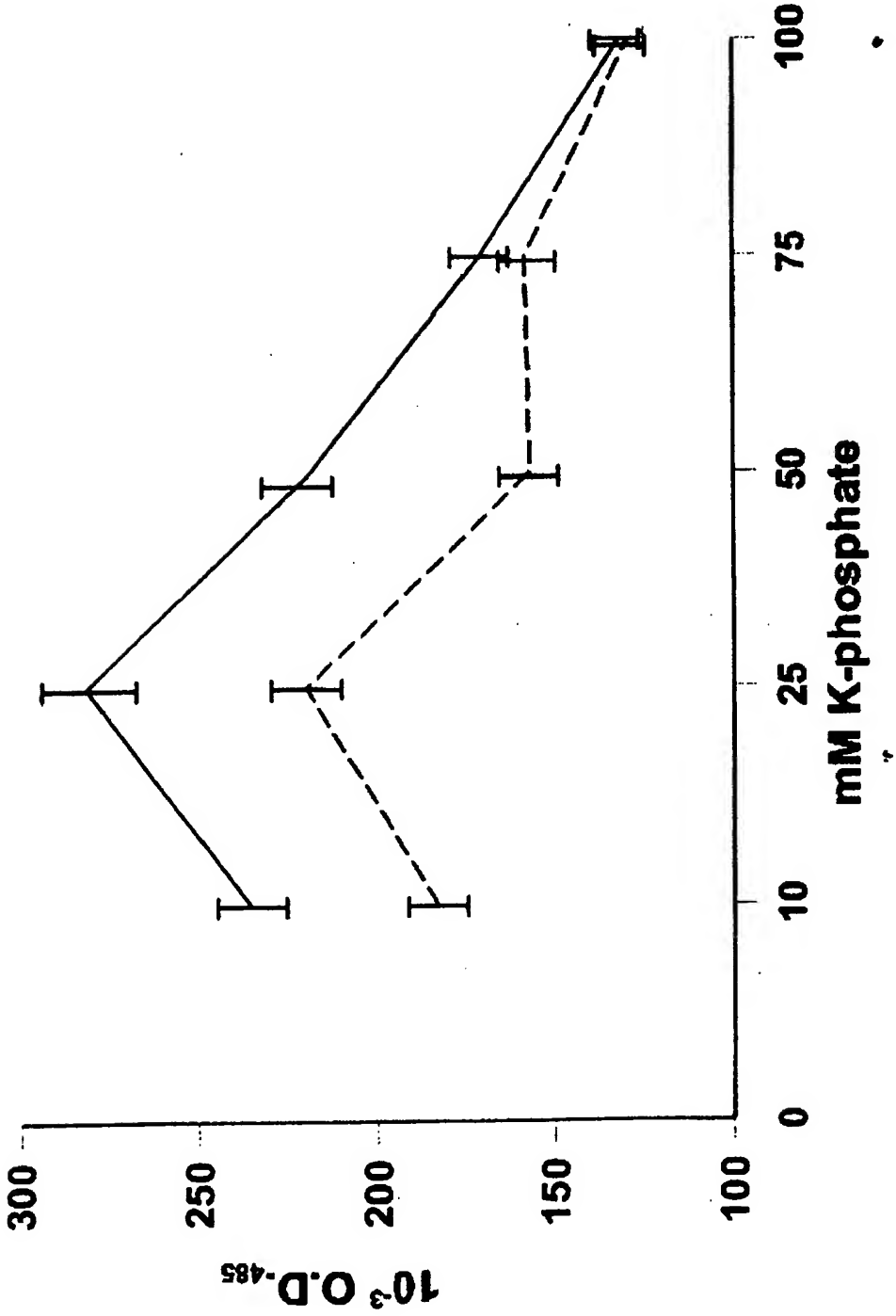
Published
Without international search report and to be republished upon receipt of that report.

(54) Title: GENETIC TRANSFORMATION USING A PARP INHIBITOR

(57) Abstract

The invention concerns a process for producing transgenic eucaryotic cells, particularly plants, which comprises: contacting a culture of untransformed cells with an inhibitor of poly-(ADP-ribose) polymerase for a period of time sufficient to reduce the response of the cultured cells to stress and to reduce their metabolism. The untransformed cells are then contacted with foreign DNA comprising at least one gene of interest under conditions in which the foreign DNA is taken up by the untransformed cells and the gene of interest is stably integrated in the nuclear genome of the untransformed cells to produce the transgenic cells. Optionally, the transgenic cells are recovered from the culture. Preferably, the inhibitor is niacinamide, preferably at a concentration of about 200 mg/l to 500 mg/l and the untransformed lls are cultured in a medium containing the inhibitor for a period of time of approximately 3 to 14 days prior to the contacting with the foreign DNA. The invention also relates to a plant having in the nuclear genome of its cells foreign DNA integrated only in the regions of the nuclear genome that are transcriptionally active in cells of the plant when the cells are treated with an effective amount of a PARP inhibitor for a period of time sufficient to reduce cell metabolism to a state where gene expression is essentially limited to genes expressed irrespective of the differentiated or physiological condition of the cell.

De Block
Supplemental Declaration
ANNEX VI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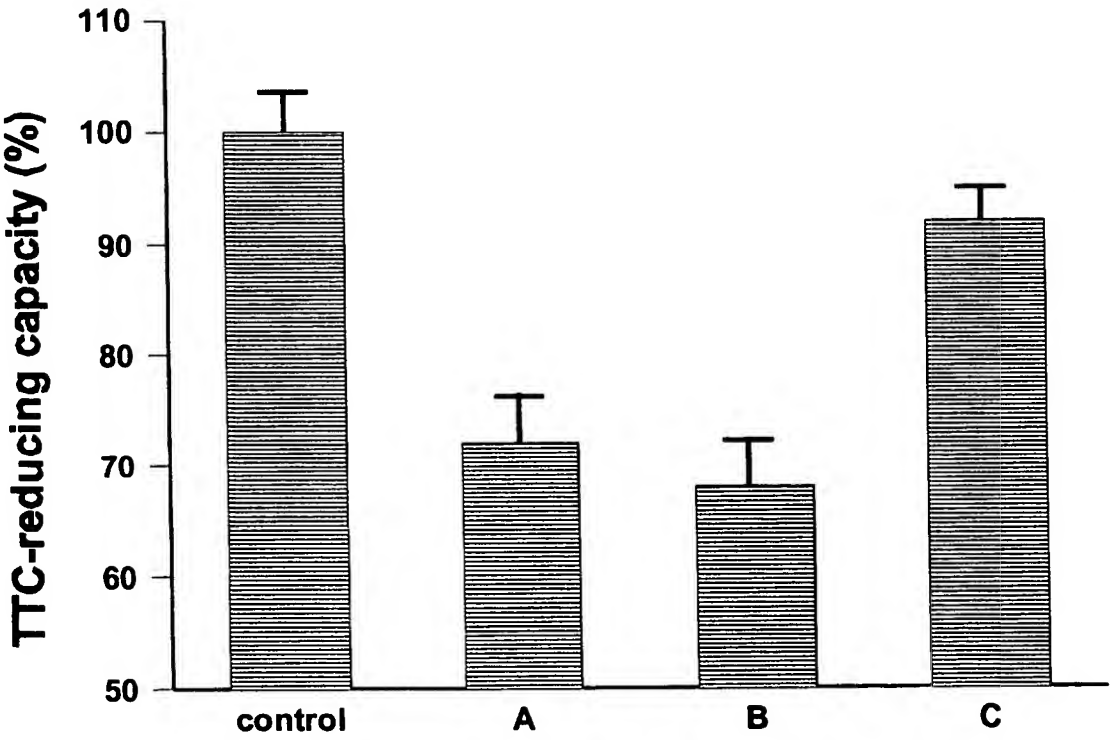
TTC-reducing capacity (%)
110
100
90
80
70
60
50
control
A
B
C